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Formulation of a live bacterial vaccine for stable room temperature storage results in loss of
acid, bile and bile salt resistance

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ABSTRACT
Live bacterial vaccines have great promise both as vaccines against enteric pathogens and as
heterologous antigen vectors against diverse diseases. Ideally, room temperature stable dry
formulations of live bacterial vaccines will allow oral vaccination without cold-chain storage or
injections. Attenuated Salmonella can cross the intestinal wall and deliver replicating antigen
plus innate immune activation signals directly to the intestinal immune tissues, however the
ingested bacteria must survive firstly gastric acid and secondly the antimicrobial defences of the
small intestine. We found that the way in which cells are grown prior to formulation markedly
affects sensitivity to acid and bile. Using a previously published stable storage formulation that
maintained over 10% viability after 56 days storage at room temperature, we found dried
samples of an attenuated *S. typhimurium* vaccine lost acid and bile resistance compared to the
same bacteria taken from fresh culture. The stable formulation utilised osmotic preconditioning
in defined medium plus elevated salt concentration to induce intracellular trehalose
accumulation before drying. Dried bacteria grown in rich media without osmotic
preconditioning showed more resistance to bile, but less stability during storage, suggesting a
trade-off between bile resistance and stability. Further optimization is needed to produce the
ultimate room-temperature stable oral live bacterial vaccine formulation.

Key words: *Salmonella* vaccines, oral delivery, formulation

Main Body:
      Conventional vaccines require refrigerated distribution and storage facilities to retain
efficacy and trained practitioners to administer injections. A 'vaccine pill' that conferred
protective immunity after oral delivery and was stable at room temperature would revolutionise
vaccination programs worldwide. One candidate technology for producing a vaccine pill is
heterologous antigen delivery by live bacteria; advanced genetic engineering techniques now
allow antigens to be stably maintained in an immunogenic form, for example the model
attenuated *S. typhimurium* vaccine strain SL3261 was modified to utilise the ORT-VAC

antibiotic free plasmid maintenance system to induce protective immunity against plague with a single oral dose [1].

The di-saccharide trehalose can be used to protect biomolecules and bacteria with water-like hydrogen bonding in an amorphous 'glassy' dry solid. In addition, osmotic pre-conditioning to induce intracellular trehalose accumulation was shown to produce dried preparations of attenuated *S. typhimurium* vaccines with exceptional stability at room temperature[2]. However, oral treatment of mice with this dried formulation was unable to protect from infectious challenge, in contrast to oral treatment with bacteria taken directly from liquid culture, suggesting that the drying protocol has a major detrimental effect on subsequent immunogenicity of live bacterial vaccines [2]. The explanation suggested for this loss of immunogenicity was a loss of invasive activity by the bacteria after drying. Enteric pathogens such as salmonella species have evolved complex mechanisms for resisting the cytotoxic effects acid and bile [3, 4], and we predicted that another possible detrimental effect of drying bacteria might be to reduce the resistance of bacteria to the harsh conditions found in the stomach or intestine after ingestion. Therefore we compared the acid and bile resistance of osmotically preconditioned dried bacteria to freshly cultured cells.

The model vaccine strain SLDAPD-pUC18I was studied, which is the attenuated *S. typhimurium* strain SL3261 modified to use the ORT-VAC plasmid-free maintenance system [1]. Cells were osmotically preconditioned in order to improve room temperature stability [2]. Osmotically stressing bacteria in rich complex broth such as LB leads to uptake of protectant molecules from the medium, therefore to induce trehalose synthesis defined or minimal medium is required [2, 5]. Briefly, cells were grown overnight with shaking at 37°C in M9 salts with 1mM MgSO$_4$, 0.1mM CaCl$_2$ and glucose to 2%, either supplemented with RPMI amino acids to 1x plus tryptophan, histidine, tryosine, and phenylalanine at a final concentration of 40mg/l each plus 2,3-dihydroxy-benzoate and para-hydroxybenzoic acid at 10 mg/l each, or enriched with 0.05% peptone; similar results were obtained with either M9 medium formulation. Osmotic preconditioning was achieved using 0.25-0.5M NaCl. Cells were harvested, resuspended in PBS with 40% trehalose plus 1.5% PVP, and then dried. The density of cells at drying was between 10^9-10^{12} cfu/ml, and within this range the cells survived drying regardless of exact density (data not shown and [2]). An initial loss of between 50-80% of cells was seen at 48h, but little further loss was seen over 50 days of storage at room temperature in a desiccator, as expected from a previous study using the same protocol to dry the parent strain SL3261 [2]. The pUC18I plasmid was maintained after dry storage for 52 days, as indicated by identical bacterial counts on replicate plates with or without ampicillin, confirming the stability of plasmids maintained using the ORT-VAC system.

As expected [3], SLDAPD taken from liquid culture showed moderate acid resistance and survived pH down to pH3.3, but were killed at pH2.6 (figure 1a). At pH of 4.2 and above, dried bacteria behaved similarly to bacteria taken from liquid culture; in contrast dried bacteria showed massive loss of viability (>1000-fold) at pH at 3.8 and below (figure 1a). The dried bacteria had no permanent loss of acid resistance, since dried bacteria given 100 minutes to recover in LB medium at 37 degrees showed identical acid resistance to the control LB culture (figure 1a). Note that in this experiment the cell densities tested differed between the dried, control and recovered bacteria, however independent experiments demonstrated that acid and bile resistance was independent of cell density over a broad range (data not shown). This reduction in acid resistance from pH 3.3 to pH 4.3 suggests that dried live bacterial vaccines

may be killed in the stomach more rapidly than fresh cultured cells.

Enteric delivery results in release into the duodenum, avoiding loss of viability in gastric acid but exposing dried bacteria directly to other defences such as bile. As expected [4], when taken from liquid cultures, SLDAPD growth was unaffected by 2% bile, although some growth inhibition was seen in 10% bile (figure 1b). In contrast, dried bacteria showed >100-fold loss of viability in 2-10% bile, and >20-fold loss of viability in 0.4% bile (figure 1b). Furthermore, dried bacteria showed >100-fold loss of viability in 3mM of the purified bile salt deoxycholine (DOC), a dose which showed no significant effect on control cells (figure 1b). The loss of bile and bile salt resistance of dried bacteria was not permanent, because dried bacteria allowed to recover in rich medium showed identical bile resistance to control cells (data not shown). Dried cells also lost resistance to 0.1% sodium dodecyl sulfate compared to control cells (data not shown), suggesting a general sensitivity to detergents. Because the intestine contains a complex mix of components, it is difficult to estimate the concentration of bile salts that bacteria are exposed to *in vivo* [4], however total bile salt concentrations in the intestine were measured in the range of 2-10mM [6]. Therefore the observed *in vitro* sensitivity to 3mM DOC raises the possibility that dried live bacterial vaccines could be killed if released directly into the intestine using enteric capsules.

To determine if osmotic preconditioning prior to drying affected bile and acid resistance of dried SLDAPD, the acid and bile resistance of osmotically preconditioned dried bacteria was compared with dried cells that had been grown in LB medium. In contrast to the osmotically preconditioned dried cells, which showed >10- to >500-fold loss of viability in bile and acid depending on the drying protocol, dried cells that had been grown in rich LB medium retained bile resistance (figure 2a, b). Control cells showed resistance to the acid and bile doses tested (figure 2c). Crucially, the improved bile resistance shown by dried cells grown in rich LB medium was accompanied with a reduced stability when stored at room temperature. LB grown cells survived the initial drying process better, with 47-84% survival at d5, in contrast to 23-26% survival at d5 for the osmotically pre-conditioned cells (figure 2d, e). However, beyond d5 the osmotically pre-conditioned cells had better stability than cells grown in rich medium. At d35 the osmotically pre-conditioned cells showed only 7-20% loss of viability compared to d5 (depending on drying protocol), and by d52 only 41-42% loss, demonstrating the high stability of these preparations (figure 2d). In contrast LB grown cells lost 64-83% live cell number at d35 compared to d5, and by d52 83-86% loss, suggesting a steady decay of these cells (figure 2e). The osmotically preconditioned cells appeared to have a decay rate approximately equivalent to a theoretical sample with a half-life of 20 days, in contrast to the LB grown cells that had a decay rate approximately equivalent to a half life of 60 days (figure 2d, e). Thus there appears to be a trade-off between optimal stability with poor bile resistance (osmotically preconditioned cells) or, alternatively, reduced stability with better bile resistance (rich medium grown cells).

Bacteria dried under high vacuum in a vacuum chamber (50mTorr at 20 degrees) had marginally less bile and acid resistance than cells dried in a desiccator under low vacuum (approx 0.1 atmosphere at 20 degrees with silica gel desiccant) with similar stability (figure 2A, B, D, E), suggesting that the drying protocol used as well as the growth conditions prior to drying affects bile and acid resistance.

The drying protocols used differed from conventional freeze-drying in that samples were dried under reduced pressure in a dry atmosphere, but temperatures were kept above freezing to

avoid freeze-injury [2]. However, it is possible that this drying protocol resulted in the observed increase in acid/bile sensitivity, and that freeze-drying would avoid it. Replicate samples of cells were frozen in parallel to drying and the stability and acid/bile resistance of frozen and dried samples compared. Compared to dried samples, freeze-thawed cells showed similar acid and bile resistance, with the exception of cells grown in rich medium that had better acid resistance after freeze-thaw than after drying (figure 2a,b). The speed of freezing had some influence on bile and acid resistance, with cells frozen rapidly in liquid nitrogen having better resistance than slower shelf freezing (figure 2a,b). The trehalose/PVP excipient provided good cryoprotection and all frozen samples showed excellent stability when stored at -80 (figure 2d,e). Since frozen cells also had lost acid and bile resistance, conventional freeze-drying will also result in dried preparations with high acid and bile susceptibility.

To determine if increased NaCl concentrations prior to drying were responsible for the loss of acid and bile resistance after drying, cells were grown in M9 with varying NaCl concentration. Increased osmolarity can reduce growth rate, and the growth stage can affect drying tolerance, so equivalent 30ml cultures were taken after 24h growth, harvested and resuspended in 0.8 ml excipient, and cell yields were compared. NaCl concentrations of 250-350 had only 2 to 4-fold reduced cell yields, but increasing NaCl to 450mM resulted in 70-fold fewer cells compared to M9 alone (figure 3f). Cells grown in NaCl concentrations of 250-350 mM also showed growth rates measured by OD600 with less than one doubling in 2h, suggesting the cells had reached stationary phase (data not shown). Cells grown in enriched M9 without added NaCl and dried showed good acid and bile resistance, whereas increasing NaCl concentration to 250-350 mM resulted in reduced bile and acid resistance (figure 3f). The reduced bile and acid resistance seen at 250-350mM NaCl is unlikely to be due to alteration in growth rates, since the final cell yield was only 2- to 4-fold reduced. Cells grown in 450mM NaCl had very poor acid and bile tolerance, however, which could be due to reduced growth rates or another effect. The reduced growth did not affect survival after drying, which was good in all cell conditions (figure 2f). Addition of NaCl up to 500mM in rich LB broth did not affect bile or acid tolerance of dried cells (data not shown).

Conclusions

We found that depending on the method used to prepare dried bacteria, drying appears to disable the resistance to acid and bile that *S. typhimurium* evolved to survive after ingestion. There appears to be a trade-off between optimal stability during storage with poor bile resistance (osmotically preconditioned cells) or, alternatively, reduced storage stability with better bile resistance (rich medium grown cells); furthermore dried cells produced in any condition showed significantly decreased acid resistance. We feel the success of current efforts to produce stable 'vaccine pills' that deliver heterologous antigens using live bacterial vectors may depend on resolving this issue of survival of the bacteria post ingestion. We are conducting further studies to elucidate the mechanism of this effect. Finally, in order to determine how the increased bile and acid sensitivity caused by drying affects oral vaccine delivery, testing of infectious doses of dried bacteria in model organisms is needed. Hopefully, these studies will result in improved formulations that improve oral survival whilst maintaining stability.

Acknowledgements

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Figure legends

Figure 1: Osmotically pre-conditioned dried bacteria show reduced resistance to acid, bile and bile salts.

A: The acid resistance of dried osmotically preconditioned cells was compared to control and recovered dried cells by incubation in LB acidified with HCl to the indicated pH for 1h at 37°C, followed by dilution and plating to determine colony forming units (cfu). Dried cells were osmotically preconditioned by overnight culture in M9 medium with 0.25M NaCl then dried in a vacuum desiccator in PBS/40% trehalose/1.5%PVP. Control cells were grown in rich medium for 4h from a glycerol stock, and recovered cells were dried cells rehydrated in rich medium and cultured for 100 minutes. Relative CFU/ml indicates the equivalent bacterial count calculated to the original volume before drying or volume of control sample before dilution in test medium; all samples were diluted at least 50x into test media to ensure dilution of the drying excipient. Note that the control and recovered cell samples were tested at a lower starting density than the dried cells, as seen in the pH7 condition; resistance to acid and bile was independent of density (data not shown). Similar loss of acid resistance by dried cells was seen in more than three independent experiments. All error bars are shown and represent standard error of more than 5 replicate colony counts.

B: The bile and bile salt resistance of dried osmotically preconditioned cells was compared to control cells by incubation in LB with the indicated concentrations of ox bile or deoxycholine (DOC) for 1h at 37°C, followed by dilution and plating. Dried cells were osmotically preconditioned by overnight culture in M9 medium with 0.3M NaCl then dried in PBS/40%

trehalose/1.5%PVP. Similar bile and bile salt resistance was seen in more than three independent experiments. All error bars are shown and represent standard error of more than 5 replicate colony counts.

Figure 2: Effect of growth condition before drying on acid or bile resistance and stability during storage.
A-E: Bacteria were either osmotic preconditioned by culture overnight in M9 medium plus 0.5M NaCl (A, D), or cultured overnight in rich LB medium (B, E), before drying or freezing as indicated and stored at room temperature in a desiccator (dried samples) or at -80 (frozen samples). **A, B:** After 5d storage, dried cells were rehydrated in LB at pH 7, LB at pH 3.8, or 5% bile and cultured for 1h followed by dilution and plating to determine live cell counts. **C**: Control cells were cultured in LB for 3h prior to testing alongside dried cells in A and B. **D, E:** Stored samples were diluted and counted before drying or freezing, or after 5, 35 or 52 days storage as indicated. Dashed grey lines indicate theoretical decay of samples with half lives of 20 or 60 days for comparison. **F:** Cells were prepared in M9 medium with the indicated concentration of NaCl before drying, then tested for acid and bile resistance for 1 hour followed by dilution and plating. The cell yield before drying is also presented, to demonstrate the effect of NaCl concentration on cell growth.